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The transcription activity of heat shock factor 4b is regulated by FGF2

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ABSTRACT

Heat shock factor 4b has been found to be closely associated with postnatal lens development. It expresses in postnatal lens epithelial and secondary fiber cells and controls the expression of small heat shock proteins which are important for lens homeostasis. However, the signal pathways underlying Hsf4b are still not completely understood. Here we present that Hsf4b transcription activity is regulated by FGF2 a key growth factor that is involved in regulating lens development at multiple stages. FGF2 can promote Hsf4b nuclear-translocation and the expression of Hsp25 and αB-crystallin, the key downstream targets of Hsf4b in the Hsf4b-reconstituted mouse hsf4−/− lens epithelial cells. Further study indicates that FGF2 can induce Hsf4b protein stabilization through ERK1/2-mediated posttranslational phosphorylation or sumoylation. Hsf4b can promote FGF2-induced morphology transition from lens epithelial cell to the fiber cell, and this morphology transition can be inhibited by ERK1/2 inhibitor U0126. Taken together, our data demonstrate that Hsf4b is a novel downstream transcription factor of FGF2, and its transcription activity is associated with FGF2-modulated lens epithelial cell–fiber cell transition.

1. Introduction

Lens development is a complex processes that is regulated by the coordinating of group of growth factors (e.g. FGFs, TGF-beta and BMP). The grown factors can determine the processes of lens development by temporospatially activating their downstream transcription factors (such as Pax6, Smads, Six3, Mab21l1, FoxE3, Prox1 and Sox2) (Garcia et al., 2011; Graw, 1996). The heat shock factor 4, which belongs to the heat shock factor family, has been found to be the fundamental transcription factor in the lens development at postnatal age. Genetic dysfunction of Hsf4b causes abnormal lens development at the postnatal age and nuclear cataract formation in mouse models (Fujimoto et al., 2004; Shi et al., 2009; Min et al., 2004), and is closely associated with human hereditary autosome chromosomal dominant cataracts (Bu et al., 2002). Hsf4 expresses in lens epithelial cell and secondary fiber cells and its transcriptional activity is regulated developmentally (Somasundaram and Bhat, 2004; Min et al., 2004). However, the regulatory relationship between the growth factors and Hsf4b transcription activity is still not understood.

Hsf4 has two isoforms, Hsf4a and Hsf4b. They are derived from the alternative splicing of mRNA between exons 8 and 9, which results in the additional 34 amino acids in Hsf4b's regulatory domain that is responsible for the varied transcription activity between Hsf4a and Hsf4b (Nakai et al., 1997; Tanabe et al., 1999). Hsf4b is the unique isoform that expresses in lens tissues (Min et al., 2004). Loss-of-function studies demonstrate that Hsf4b is involved in transcriptionally up-regulating the expression of small heat shock proteins (e.g. Hsp25, γ-crystallin and αB-crystallin) and skeletal filaments (e.g. Bead filensin, CP49 and SKAP2) but negatively regulating the expression of FGF-4 and FGF-7 in lens tissues (Min et al., 2004; Zhou et al., 2011; Shi et al., 2009; Fujimoto et al., 2004). Hsf4b is a phosphorylation-regulated protein (Hu and Mivechi, 2006). Our previous investigation indicates that Hsf4b can associate with MAP kinases (ERK1/2, JINK and P38). Phsphorylation of Hsf4b by ERK1/2 and P38 causes Hsf4b protein stabilization and transcriptional activation. Erk1/2 phosphorylation can be inhibited by Dusp26 by forming a complex of Hsf4-ERK1/2-DUSP26 (Hu and Mivechi, 2006). Furthermore, we found that Hsf4b can inhibit CMV promoter activity, and its inhibitory function is regulated by the phosphorylation of Hsf4b/S299 and its associated protein Daxx. Daxx can associate with and inhibit Hsf4b transcription activity, and their association is regulated by the phosphorylation of Hsf4b/S299 (Zhang et al., 2010). Hsf4b/S299 is a key amino acid of the PDSM motif (phosphorylation -dependent sumoylation motif) which is conserved in many transcription factors with transcriptional inhibition activity (Hietakangas et al., 2006). Hsf4b/S299 also consists of the MAP Kinase phosphorylation motif (PxSP) (Clark-Lewis et al., 1991).

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Therefore, we hypothesize that MAP Kinases may be involved in regulating the Phosphorylation-dependent-sumoylation. Investigation of the regulatory mechanism of MAP kinase and their upper signal pathways (e.g. growth factors) will help to understand the signals underlying Hsf4b during lens development.

FGF2 is an important growth factor in lens development. It is involved in lens placode formation, epithelial proliferation and the primary and secondary fiber cell differentiation (Yang et al., 2006; Belecky-Adams et al., 2002; McAvoy et al., 1991). *In vitro* studies suggest that low concentrations of FGF2 can induce lens epithelial cell proliferation while high concentrations of FGF2 can promote epithelial cell differentiation (Lovicu et al., 1997; Lovicu and McAvoy, 2001). FGF2 can activate the MAP Kinase/ERk1/2 and PI3K-AKT pathways leading to the activation of the transcriptional factors pax6, Sox2, and FoxE3 and the expression of alpha A-crystallin and alpha B-crystallin, which are important for lens placode formation and primary fiber cell differentiation (Yang et al., 2006; Faber et al., 2001). FGF2 can also inactivate the transcription factor L-MAF through ERK1/2 phosphorylation-induced degradation (Ochi et al., 2003), which is important for chicken lens epithelial cell proliferation. However, whether Hsf4b transcription activity is regulated by FGF2 is still not clear.

In this paper, we find that Hsf4b transcription activity is regulated by FGF2. FGF2 can induce Hsf4b protein sumoylation and stabilization, and enhance Hsf4b nuclear translocation and transcription activity by activating the MAP kinase ERK1/2 pathway. Furthermore, we found that Hsf4b participates in the regulation of FGF2-induced lens epithelial cell transition to the fiber cells. Our data indicate that Hsf4b is a novel downstream target of FGF2, and its transcription activity is associated with FGF2-induced morphology switching from lens epithelial cell and fiber cell.

2. Experimental procedures

2.1. Cell culture and reagents

The cell lines mLEC/hsf4−/− (Zhang et al., 2010), 293-pheonix (used for retroviruses package) and HEK293 were routinely grown in DMEM media containing 10% FBS, 100 μM/ml ampicillin–streptomycin. The kinase inhibitors U0126, SB203580 and wortmannin are from Cell Signaling, USA. The mouse basic FGF2b is from Sigma, USA.

2.2. Plasmids and retroviral infection

Plasmids: pWZL-HA-Hsf4b and pWZL-HA-CRYAB. The N-terminal fused HA-tag of human Hsf4b and mouse CRYAB cDNAs are subcloned at EcoRI and BamHI of pWZL-blasticistin vector respectively; pWZL-Hsp25: the mouse Hsp25 cDNA was subcloned into the BamHI and XhoI sites of the pWZL-blasticistin vector. pGL-CRYAB-luciferse: a 960 bp DNA fragment spinning upstream of ATG in the CRYAB genome was amplified from the mouse genomic DNA (C57BL/6 strain) with PCR, and subcloned into the pGL2-basic vector at the restrictive enzyme sites of MluI and BglII. All recombinant plasmids were verified with DNA sequencing. For the retrovirus infection, the recombinant retrovirus vectors were transiently transfected into the 293-phoenix cells for retrovirus packaging. The mLEC/hsf4−/− cells were infected with the retrovirus and then selected with 3 μg/ml Blasticidin for 3 days. The survived cells were pooled together and maintained in media containing 1 μg/ml blasticidin.

2.3. Quantitative RT-PCR

Total RNA extracted with TRIzol reagents were used to synthesize the first strand of cDNA with the cDNA synthesis kit (Promega, USA). For the semi-quantitative RT-PCR, the cDNA was diluted at 1:50. The PCR was performed using 25 cycles for amplification of the cDNAs of Hsp25, CRYAB and actin. For the quantitative real time PCR, the prepared cDNAs were mixed with SYBR–PCR mixtures with proper primers and amplified in the BioRad CFX96 machine (BioRad, USA). The quantity was counted from three independent experiments.

2.4. Immunoblotting, immunoprecipitation, and GST-pull down

Cells were lysed in modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.25% DOC, 0.5% NP-40 and 1× protein inhibitor cocktail and 1× phosphatase inhibitor cocktail (Sigma)). The protocols for immunoblotting, immunoprecipitation, and *in vivo* GST-pull down have been published previously (Zhang et al., 2010). The antibodies against Hsp25, Hps90, Hsp70 and P23 were from Stressgen, USA. The antibodies against αB-crystallin, Hsf4 and β-Actin were from Santa Cruz Biotech, USA; the anti-HA-tag antibody was from Novus, USA, and the antibodies against p-AKT, AKT1, ERk1/2, phospho-Erk1/2, P38 and phosphor-p38 were from Cell Signaling, USA.

2.5. CHIP assay

The assay was performed according to the protocol of the kit (Upstate, USA). Briefly, 1×10^7 mLEC/hsf4−/− mock and mLEC/HA-Hsf4b cells were incubated with 1% formaldehyde for 10 min at 37 °C followed by two PBS washes. The cells were lysed in SDS lysis buffer and sonicated to break up the chromosome DNA into 200–1000 bps. After diluting with the diluted buffer and pre-cleaned with 50% slurry protein-A agarose beads containing sheared salmon DNA, the cell lysates were incubated with anti-HA antibody at 4 °C overnight followed by incubation with 50% slurry protein-A-agarose beads containing sheared salmon DNA for 4 h. The protein-A agarose beads were washed once with low salt buffer, high salt buffer and TE buffer. The protein–DNA complex was eluted in elution buffer. The protein–DNA complex was converted at 56 °C for 4 h and incubated with proteinase K at 50 °C for 1 h. The DNA fragments were extracted with phenol–chloroform, and subjected to PCR with Primers covering the promoter of CRYAB, and Hsp25. GAPDH is used as a negative control.

2.6. Chase assay

The mLEC/Mock and mLEC/HA-Hsf4b cells, which were treated with sham (PBS) or with FGF2 for 12 h, were incubated with 30 μg/ml of cycloheximide for the proper time. The cells lysates were subjected to immunoblotting with the proper antibodies.

2.7. Immunofluorescent staining and luciferase reporter assay

mLEC/HA-Hsf4b cells were treated with sham or FGF2 for 12 h, and then subjected to immunostaining with anti-HA antibody followed by the cys3-conjugated anti-mouse secondary antibody. The nuclei were stained with Dapi. The signals were photographed under the Zeiss 540 microscopy under 100 indexes. For luciferase, the construct pGL2-P-cryab promoter was transiently cotransfected together pcDNA-beta-Gal into the mLEC/Mock and mLEC/HA-Hsf4b cells. The cells were left without any treatment or treated with 100 ng/ml FGF2 for 12 h. The luciferase value divided